

The Hedgehog Pathway in Triple Negative Breast Cancer

A Thesis

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Abstract

Triple negative breast cancer (TNBC) is a common subtype of breast cancer comprising about 15% of the 230,000 new breast cancer cases every year. TNBC is named due to the lack of three common receptors that are prevalent in most breast cancer: Estrogen Receptor α (ER α), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor (HER2). Many breast tumors are induced through estrogen and ER α , but these can be treated with endocrine therapy which inhibits ER α . The lack of ER α and the other receptors limit drug options to chemotherapy regimens which tumors often develop resistance against. Consequently, it is important to identify new pathways involved in TNBC and drugs that can provide therapy to these patients. One such pathway is the Hedgehog (Hhg) pathway which is upregulated in a number of cancers like basal cell carcinoma and medulloblastoma. The Hhg pathway is a highly conserved pathway that is important for embryonic development and in controlling cell proliferation and differentiation. Due to the Hhg pathway's selective activation in adult cancer cells, it is an ideal candidate for therapeutic intervention. We believe that the Hhg pathway plays an important role in TNBC development and can be targeted therapeutically in these patients. Here we show that in primary human breast tumors and in TNBC cell lines MDA-MB231, MDA-MB468, and BT20 that the Hhg pathway is activated and that it may be regulated in part through the PI3K/AKT pathway. We also show that the very promising anti-Hedgehog compound GDC-0449, already approved for basal cell carcinomas, can inhibit TNBC cell growth in combination with PI3K inhibitor. We intend to test the efficacy of GDC-0449 in combination with PI3K inhibitors *in vivo* to provide a novel targeted therapy for TNBC patients.

Table of Contents

Problem statement.....	pg. 4
Background.....	pg. 4-11
Figure 1.....	pg. 6
Figure 2.....	pg. 8
Significance.....	pg. 10
Hypothesis.....	pg. 10
Objectives.....	pg. 11-14
Figure 3.....	pg.13
Methodology and Rationale.....	pg. 15-17
Figure 4.....	pg.17
Background data.....	pg. 17-20.
Figure 5.....	pg. 19
Figure 6.....	pg. 19
Figure 7.....	pg. 20
Results.....	pg. 20-27
Figure 8.....	pg. 22
Results 9	pg. 23
Figure 10.....	pg. 26
Figure 11.....	pg. 27
Discussion.....	pg.27-30
Figure 12.....	pg. 30
Acknowledgements.....	pg.30

Problem

Triple negative breast cancer is one of the most deadly subtypes of breast cancer that accounts for ~15% of the total breast cancer cases and is especially prevalent in the Latino, black, and obese populations. The exact mechanism for the initiation and progression of this deadly breast cancer subtype has not been established. The purpose of this study is to investigate the potential role of the Hedgehog pathway in this process. This pathway is responsible for cell differentiation and body segmentation. It is normally silenced by adulthood but can be reactivated in cancers where it contributes to cell proliferation, metastasis, and the maintenance of cancer stem cells. We and others have previously shown that the Hedgehog pathway is activated in multiple triple negative breast cancer cell lines as well as human breast tumor samples. We have suggested a probable mechanism activating the pathway in these cells and a possible therapeutic strategy in patients with triple negative breast cancer.

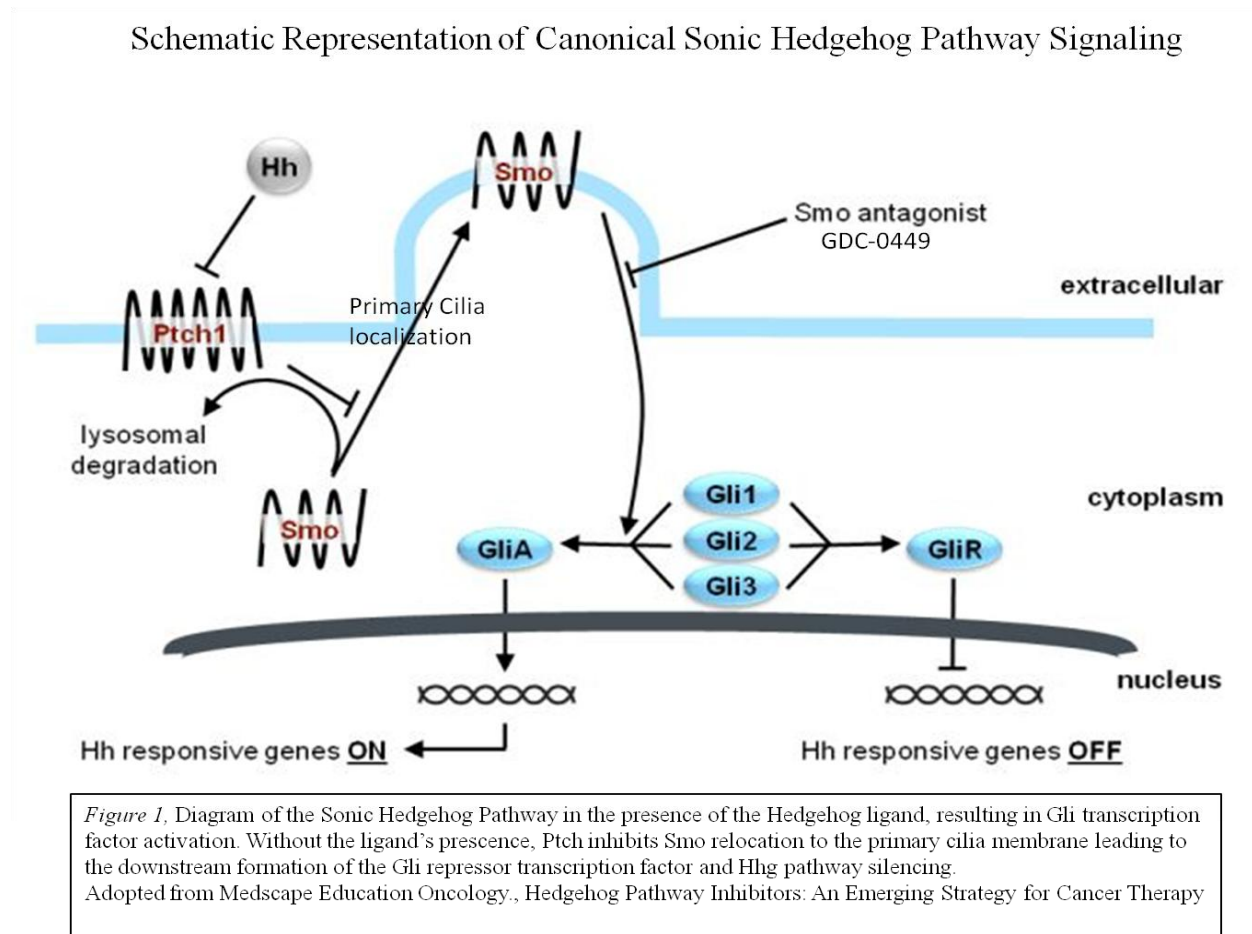
Background

Breast cancer and the Triple negative subtype

Breast cancer is the second most common cancer among women with 230,480 new cases of invasive breast cancer and 39,520 deaths in 2011². About 30% of the early diagnosed cases will have a recurrence and 50% already have or will develop resistance to chemo or endocrine therapy. Triple negative (TN) breast cancer is a common subtype of breast cancer comprising about ~15% of the total cases and is especially prevalent among the African, the African-American, the Latino, and the obese populations¹. TN breast cancer comprises several sub-types of breast cancer including basal-A and basal-B breast cancer³ and more rare forms like metaplastic tumors and adenoid-cystic tumors⁴. TN breast cancer is named due to the lack of

expression of three common receptors that are frequently associated with most breast cancer: Estrogen Receptor α (ER α), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2). Estrogen promotes tumor growth by virtue of its non-genomic (growth factor signaling through ER α) and genomic effect (transcriptional regulation by ER α). Growth of ER α positive breast tumors are stimulated by estrogen and therefore can be treated with anti-estrogenic therapy like Tamoxifen, which binds to and inhibits ER α function. Other treatment options for breast cancer besides endocrine therapy include surgery, radiation, and chemotherapy which are often combined in patient treatment. But the lack of ER α and other growth factor receptors in TN tumors limit drug treatment options to chemotherapy that often leads to the development of resistance. Consequently, it is important to identify new pathways critical for growth and survival of TN cancers and develop targeted therapy for these patients. One such pathway is the Hedgehog (Hhg) pathway which we show to be upregulated in TN breast cancer cell lines (see background data).

Figure1



The Hedgehog Pathway

The Hh pathway is a highly conserved and important embryonic developmental pathway that controls cell proliferation and differentiation. By adulthood, the pathway is largely inactive other than in stem cell populations and maintaining tissue homeostasis⁵. Activation of the pathway has also been heavily implicated in cancer and has been shown to be especially prevalent in basal cell carcinoma, medulloblastoma, and pancreatic cancer⁶. More recently, activation of Hh pathway has been demonstrated in many solid tumors such as glioblastoma, melanoma, breast, lung, and prostate cancers⁷. The pathway is activated mainly through two

different mechanisms: the ligand dependent (canonical) pathway and the ligand independent (non-canonical) pathway.

The ligand dependent pathway involves the inhibition of the membrane bound receptor PTCH1 through the Sonic, Desert, or Indian Hedgehog ligand. The inhibition of PTCH, allows the release of the G-protein coupled receptor Smoothened (SMO) which in turn activates the GLI zinc-finger transcription factors. GLI1 is solely a transcriptional activator, while GLI3 and GLI2 can act as activators or repressors depending on post-translational modifications⁸ (pathway conceptualized in Figure 1⁹). Much of the activity of the pathway in normal vertebrae signaling is dependent on a sensory organelle called the primary cilium, discussed in detail later. When activated, the GLI proteins upregulate expression of multiple target genes and induce proliferation, epithelial mesenchymal transition, angiogenesis, metastasis, and activation of other cancer stem cell pathways¹⁰⁻¹³.

The ligand independent pathway involves the activation of GLI1 and GLI2 downstream of PTCH, where binding of hedgehog ligands to PTCH is not important (discussed in detail later). In the absence of Hhg pathway activators, the repressor form of GLI3 predominates and inhibits transcription of Hhg target genes. Aberrant activation of Hhg signaling has been shown in basal cell carcinomas and medulloblastomas with mutations in *PTCH1* and *SMO*¹⁴⁻¹⁵.

The Hhg pathway plays an important role in mammary development but is largely inactive in adult tissue. Severe ductal morphogenesis and dysplasia results when *Ptch1* is disrupted in mice¹⁶. Furthermore it has been shown to play an important role in promoting multiple drug resistance via efflux of the drug by an ABC transporter dependent mechanism¹⁷.

Figure 2

Hedgehog Signaling within the Primary Cilium

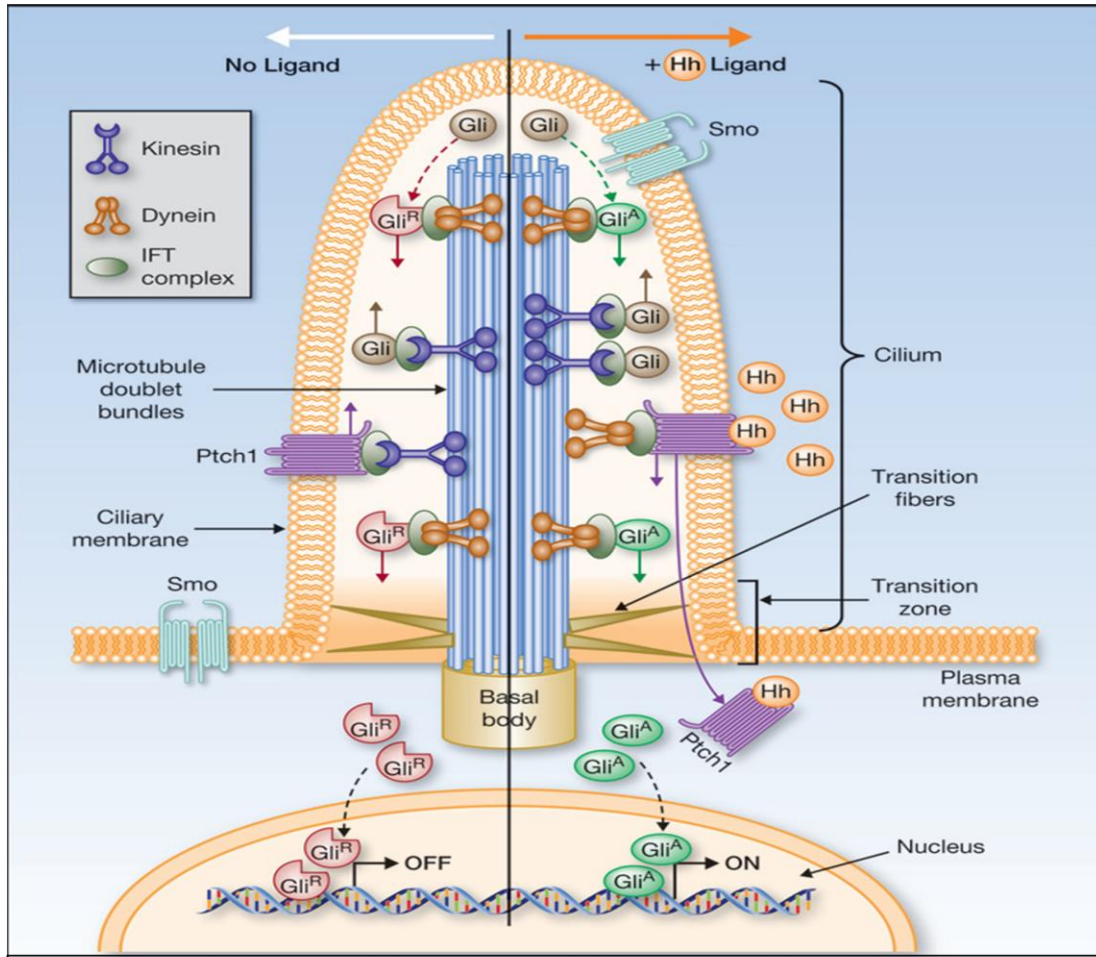


Fig 2. The primary cilium plays an important role within the context of Hedgehog activation. Without extracellular ligand activation, the pathway is non-functional as SMO remains on the cellular membrane as PTCH is able to inhibit its translocation to the ciliary membrane. Without SMO, the GLI family transcription factors are processed into the repressor forms (GLI2 and GLI3) and Hedgehog target genes are silenced. Under the presence of the Hedgehog ligand, PTCH activity is abolished, allowing for the migration of SMO into the cilium. Smo then acts through a cell signal cascade to ultimately allow the GLI family to be modified into the transcriptionally active forms. Adopted from Hassounah NB et al. Clin Cancer Research 2012.

Primary Cilia in the context of Hedgehog and Cancer

Activation of Hhg pathway depends on trafficking of key signaling molecules (such as PTCH, SMO, and GLI) to primary cilium which is a sensory organelle common to epithelial

cells in vertebrates. Primary cilia have a continuous yet distinct membrane from the plasma membrane and have transition fibers at the base of the cilia to prevent passive diffusion between the cilium and rest of the cytosol¹⁶. This exclusion from the cellular compartment allows the primary cilia to be sensitive to the environment without interfering in normal cellular processes. Kinesin2 and Dynein are two important proteins involved in protein trafficking along the microtubules, which are essential for primary cilium dependent signal transduction pathways like Hhg^{12,18,19-20}. In ligand dependent Hhg signaling, PTCH, SMO, and the GLI transcription factors undergo post-translational modifications predominantly within primary cilia (Figure 2)¹⁸. In the presence of Hh ligand, PTCH is recruited to the primary cilium membrane followed by similar translocation of SMO to the primary cilium compartment. Suppressor of Fused (SUFU), a key repressor of GLI sequesters GLI proteins within the cell cytoplasm in absence of the Hhg ligands. After activation of Hh pathway via ligand binding to PTCH, GLI proteins translocate into the primary cilium where the SUFU-GLI complex is disassociated, resulting in GLI activation.

After seeing the importance of the cilia in the context of Hhg signaling, many researchers have recently looked into primary cilia expression in human cancer patients. Primary tissue samples from renal cell carcinoma²¹, melanoma²², basal cell carcinoma²³, medulloblastoma²⁴, and pancreatic cancer²⁵ patients all showed reduction in primary cilia when compared to adjacent normal tissue. In breast cancer, the issue of primary cilium is more complex as a study of primary tumors and cell lines of various breast cancer subtypes showed differences in primary cilia expression. The basal-B breast cancer subtype, which are TN tumors by definition, were shown to be the only breast cancer type to have high amounts of primary cilia in cancer cell lines (MDA-MB 231, MDA-MB 435, SUM1315, and Hs578T)²⁶.

Non-canonical Signaling of the Hedgehog Pathway

In recent years it has come to light that the Hhg pathway can be activated in the absence of Hhg ligands via cross-talk with other signaling pathways such as the PI3k/AKT and the MEK1/2 pathways²⁷⁻²⁸. The PI3k/AKT pathway plays a very important role in protecting Gli1 and Gli2 transcription factors from degradation by inhibiting Protein kinase A. The MEK1/2 pathways play an important role in stabilizing Gli1 and Gli2 via MEK1 binding to the N-terminal end of the transcription factors. Although the detailed mechanism remains unknown the stabilization caused by the MEKs is thought to facilitate nuclear translocation of Gli. Both PI3k/AKT and MEK1/2 pathways are highly active in TN breast cancers⁸ making these pathways very intriguing areas of further study.

Hedgehog and Cancer Therapeutics

As the Hhg pathway is predominantly activated only in cancer cells in adults, it is an ideal candidate for therapeutic intervention. Genentech has recently developed a small molecule anti-Hedgehog compound named Vismodegib (GDC-0449). By inhibiting SMO, GDC-0449 blocks activation of GLI1 and downregulates its target genes. It has already been used in Phase I and Phase II clinical trials on patients with advanced basal cell carcinoma showing clinical benefit in almost all patients. Last January, GDC-0449 was approved for a Phase III trial by the FDA and therefore, could be a therapeutic option for many cancer patients. GDC-0449 has minimal side effects when taken orally²⁹. Therefore, this small molecule inhibitor could be used to target the Hhg pathway in patients with triple negative breast cancers. Several other pharmaceutical companies are also developing SMO inhibitors based on the success of GDC-0449 in the clinical trials.

Significance

Triple negative breast cancer continues to be one of the most deadly subtypes of breast cancer especially among the young and minorities. Late detection and lack of targeted therapies largely contribute to the poor prognosis of the patients with TN breast cancer. The Hedgehog pathway presents a possible alternative therapeutic target that could provide immediate survival benefits to patients with TN breast cancer. With the success of anti-Hhg compounds in the clinic like Genentech's GDC-0449 and other anti-Hedgehog compounds ready for clinic trials, targeting the Hhg pathway in cancer has become an area of intense therapeutic research. By dissecting the complex mechanism of Hhg activation we hope to gain new insights into the crosstalk between Hhg and other oncogenic pathways that could in the future be used for combination therapy in conjunction with anti-Hhg compounds.

Hypothesis

We and others have already shown that the Hhg pathway is activated in TN breast cancer cell lines and primary human tumors¹¹ (see background data). Based on our own data as well as others we hypothesize that altered expression of Gli1 in TN breast cancer cell lines, due to a complex crosstalk with other oncogenic pathways, is contributing to increased Hedgehog signaling and therefore tumorigenesis. Furthermore, silencing this pathway could provide therapeutic benefits to patients.

Objectives:

Specific aim 1: Decipher the mechanism underlying the upregulation of the Hedgehog pathway in triple negative breast cancer by exploring potential cross-talk with other signaling pathways.

Our group (manuscript submitted) and others have shown a cross-talk between the PI3k/AKT and Hhg pathway that results in activation of Hhg¹¹. Because of high PI3k/AKT activity in TN breast cancer, there is a strong rationale to delve deeper into the possible cross-talk between the two pathways and determine if any other signaling mechanism contributes to Hhg activation in TN breast cancer.

Specific aim 2: Identify key downstream targets of Gli1 contributing to tumorigenesis.

In other cancers, several downstream targets of the Hedgehog pathway, involved in metastasis, proliferation, stem cell characteristics, and angiogenesis have been identified. We will look at known targets of the Hhg pathway as well as other genes within the Hhg pathway to further characterize the pathway within TN breast cancer.

Specific aim 3: Assess the efficacy of anti-Hhg compounds in terms of cancer therapeutics.

In recent years, the Hhg pathway has been shown to be an ideal target for developing small molecule inhibitors and many pharmaceutical companies have manufactured targeted therapies against SMO and GLI1. We will test the efficacy of some of these compounds as well as the endogenous repressors of the pathway in TN breast cancer.

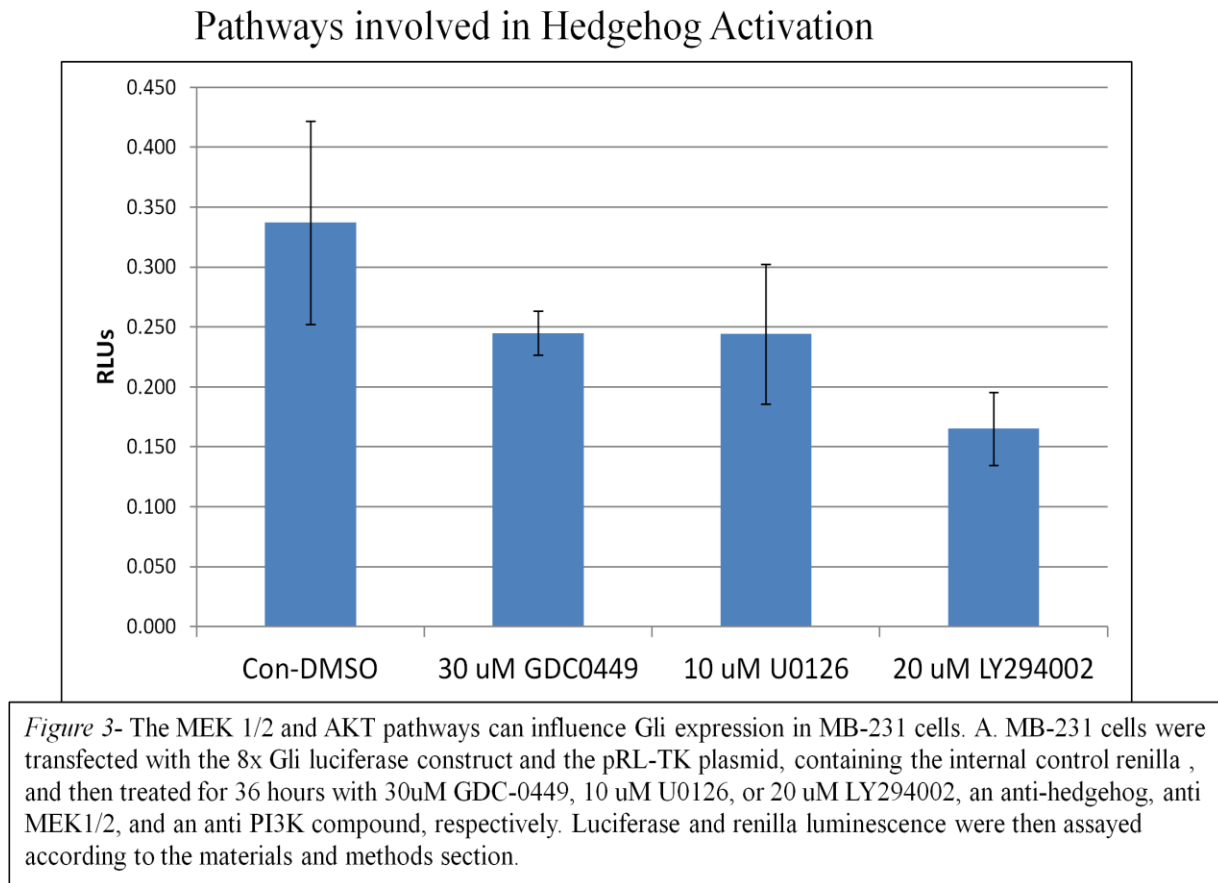
Study Design

Specific aim 1: Decipher the mechanism behind the upregulation of the Hedgehog pathway in triple negative breast cancer by exploring the potential cross-talk with other signaling pathways.

We have previously shown the importance of the PI3K/AKT pathway in regulating GLI transcription factor (Figure 3), making it a strong candidate for further investigation. It is

therefore, important to study if a cross-talk between the two pathways could regulate the aggressive nature of TN breast cancer. Using common TN cell lines, MDA-MB231 and MDA-MB-468, we will further explore the relationship between these pathways as outlined below.

Figure 3



Confirming the role of the PI3K/AKT pathway in TNBC Hedgehog Signaling

To confirm the role of PI3k/AKT in activating Hhg signaling, we will use a constitutively active (overexpression) and dominant-negative mutants of PI3k and AKT¹⁹. These plasmids will be transfected into TN breast cancer cell lines and the transactivation potential of GLI will be analyzed using the 8x GLI-luciferase plasmid (described in materials and methods). We will also

use the PI3k/AKT inhibitor LY2940002 to test whether its inhibitory effect could be rescued by overexpression of constitutively active PI3k/AKT. This will demonstrate the specificity of PI3k/AKT in regulating Hedgehog pathway. Upon completion of these experiments we will further analyze the downstream signaling events that regulated Gli1 activation through the PI3k pathway.

Specific aim 2: Identify key downstream targets of Gli1 contributing to tumorigenesis.

Using Real-time PCR, we aim to identify genes differentially expressed between TN cell lines and controls. We will test several known targets of the Hhg pathway involved in tumorigenesis as well as other genes involved within Hhg in order to characterize the pathway in TN breast cancer cells further.

Specific aim 3: Assess the efficacy of anti-Hhg compounds as well as the endogenous suppressor of Hedgehog pathway in terms of cancer therapeutics.

We first will determine the effect of anti-Hhg compounds on TN breast cancer cell lines *in vitro*. Based on these studies, we hope to use these compounds to attenuate the growth of tumors induced in mice with TN breast cancer cells. This could lead to clinical trials in patients with TN breast cancer in the future. We will focus our attention on the small molecule inhibitor of SMO, GDC-0449; the small molecule inhibitor of GLI1, GANT61³⁰.

Methods

Tissue culture

Established triple negative breast cancer cell lines, MDA-MB 231, MDA-MB 436, and MDA-MB 468 were cultured in DMEM with 10% fetal bovine serum (FBS) (Sigma Aldrich).

Triple negative BT-20 cells were cultured in MEM and 10% FBS, while ER α positive cell lines T47D and BT474 were cultured in RPMI 1640 with 10% FBS. The ER α positive cell line MCF7 was cultured in DMEM with 5% FBS and 6ng/mL insulin. The MCF10A, normal breast epithelium cell line established from a triple negative breast cancer patient, was cultured in DMEM/F12 media supplemented with 10% FBS, cholera toxin, insulin, hydrocortisone, and EGF. All media and supplements were obtained from Sigma Aldrich and cells were cultured at 37°C in a 5% CO₂ saturated incubator.

RNA and Protein

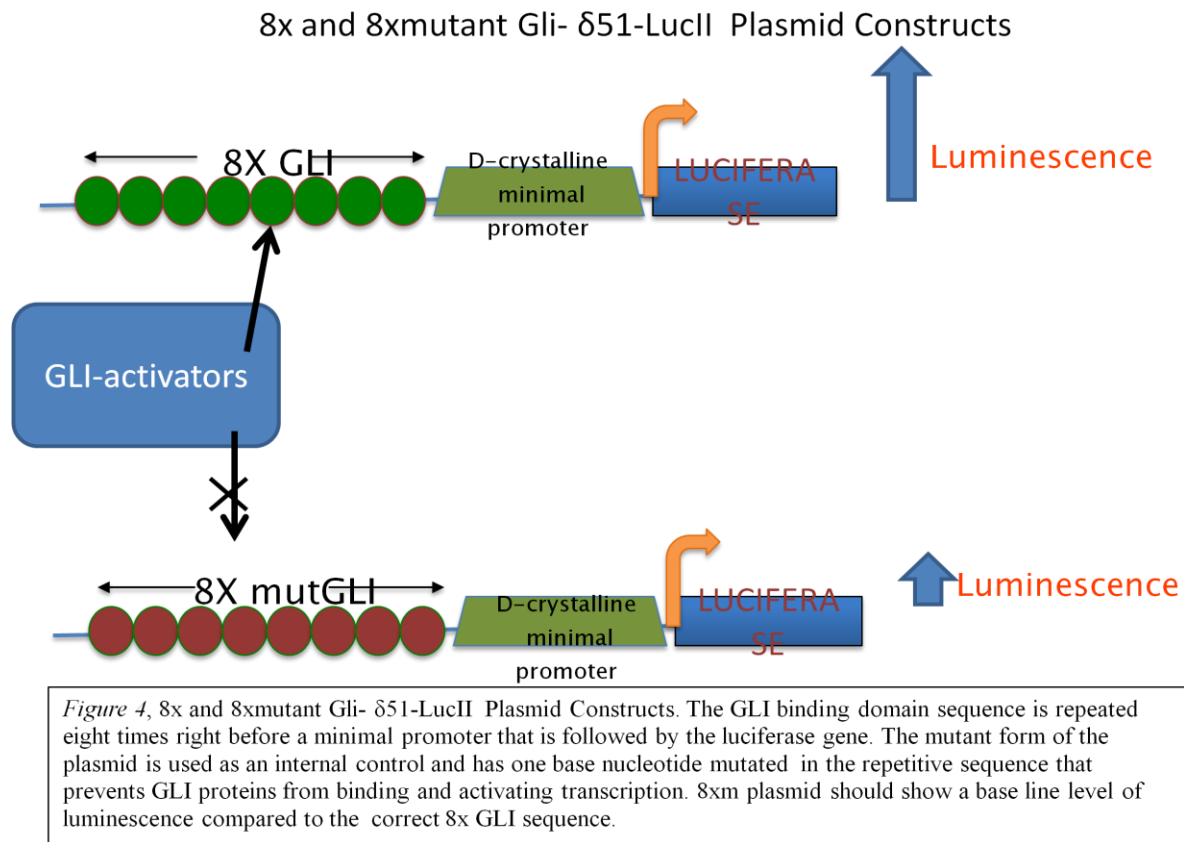
Cells were grown to 70-80% confluency, washed with 1x PBS and then harvested for RNA isolation using Trizol (Sigma) or for protein extraction. RNA was isolated and cDNA made using High Capacity cDNA kit (AB Biosciences). Real-time PCR was performed with the cDNA to assess mRNA levels in the cells by using Taqman assays (AB Biosciences) and SYBR-Green chemistry (Sigma) (primer sequences available upon request.) Semi quantitative PCR amplification was performed with the cDNA and the products were visualized on agarose gels.

Protein was extracted using cell lysis buffer, sonicated and used for western blots. Protein was separated on SDS-PAGE gels and then transferred to a nitro-cellulose membrane and probed for proteins of interest using specific antibodies. Anti- α/β Tubulin rabbit polyclonal antibody (#2148) was obtained from Cell Signaling Technology, Gli1 mouse monoclonal antibody (SC-271075) was bought from Santa Cruz Biotechnology, Smoothed (Smo) rabbit polyclonal antibody was from Abcam (Ab38686).

Transfections and Luciferase Assays

Cells were transfected using Lipofectamine 2000 (Sigma Aldrich) in antibiotic free media at 75% confluency and the media was changed 16 hours post-transfection. 8x-Gli- δ 51-LucII and 8xm-Gli- δ 51-LucII were generous gifts from Dr. Hidenao Sasaki of Hokkaido University, Japan³¹. The plasmid allows for the quantification of the activity of the Gli1 transcription factor. Eight repeats of the Gli1 binding sequence is cloned in front of the minimal promoter of the δ -crystalline gene that drives the expression of luciferase from the plasmid. This allows the Gli1 transcription factor to bind to the sequence and activate the transcription of luciferase which can be quantified using the Dual Luciferase Assay kit (Promega) (see figure 4). To ensure that the luciferase expression is due to Gli-mediated activation, we used an identical plasmid where Gli1 binding site was mutated. The pRL-TK vector where renilla luciferase expression is driven by thymidylate kinase promoter was also transfected into the cells, which served as the internal control. Cells were harvested at times varying from 36-48 hours after transfection (see experiments for specific time). GDC-0449 was purchased from Dr. Ching Shih-Chen's lab at The Ohio State University while U0126 and LY2940002 were purchased from Sigma Aldrich. Drugs and/or ligands were added to the transfected cells 16 hours post-transfection. Recombinant Sonic Hedgehog Ligand was obtained from R&D Systems.

Figure 4



MTT Assays

In vitro cell proliferation assays were carried out according to the MTT assay protocol provided with the kit (Roche). MB-231, MB-436, and MB-468 cells were seeded in 96 well plates, starved, and then treated with drugs for varying lengths of time. Cell growth is quantified by using a spectrophotometer to measure absorbance in the wells. All assays were done in quadruplicate.

Background Data

The Hedgehog Pathway is activated in Triple Negative Breast Cancer Cell Lines and Correlates with Poor Disease Free Survival in ER α Negative Patients

TN breast cancer cell lines BT-20, MB-231, and MB-468 and ER α positive cell lines T47D and ZR 75.1 were harvested and protein extracted for Western blot analysis. All three TN breast cancer cell lines showed significantly higher level of SMO expression, compared to the ER α positive cell lines (Figure 5a). Protein was normalized to α/β Tubulin and quantified using ImageJ software (Figure 5b.). We also found GLI1 to be highly expressed in the TN cell lines when compared to the ER α positive cell line OHTR (Tamoxifen resistant MCF7 cells) and normal breast epithelium MCF10A cells (Figure 6a). Protein was again normalized to α/β Tubulin (Figure 6b). Total RNA was isolated from all the TN cell lines and MCF7 cells (an ER α positive cell line), and GLI1 expression was analyzed using Taqman Real-Time PCR and normalized to β -actin. The data showed significant upregulation of GLI1 in the TN cell lines compared to MCF7 cells (Figure 6c).

In collaboration with Dr. Bhuvaneswari Ramaswamy, we were also able to show that high Gli1 expression in breast cancer patients with estrogen receptor negative disease correlates significantly with poor disease free survival (Figure 7) [n=230]. Tissue microarray prepared from Patients with TN breast cancer were assayed using immunohistochemistry for Gli1 expression, scored by pathologists, and scored for high and low Gli1 expression.. About 60% of the patients in the cohort were in the high Gli1 expressing subgroup. This data strongly highlights the importance and relationship between Hedgehog signaling and poor patient outcome and shows that Hedgehog status could be an important prognostic marker for patients with TN breast cancer.

Figure 5

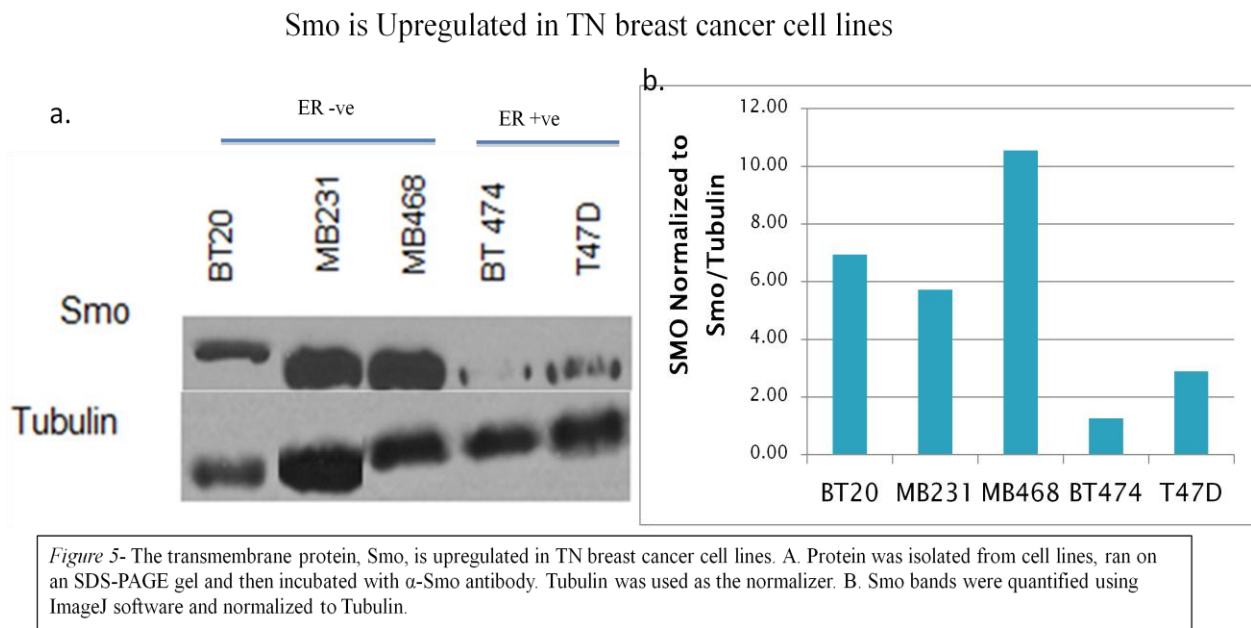


Figure 6

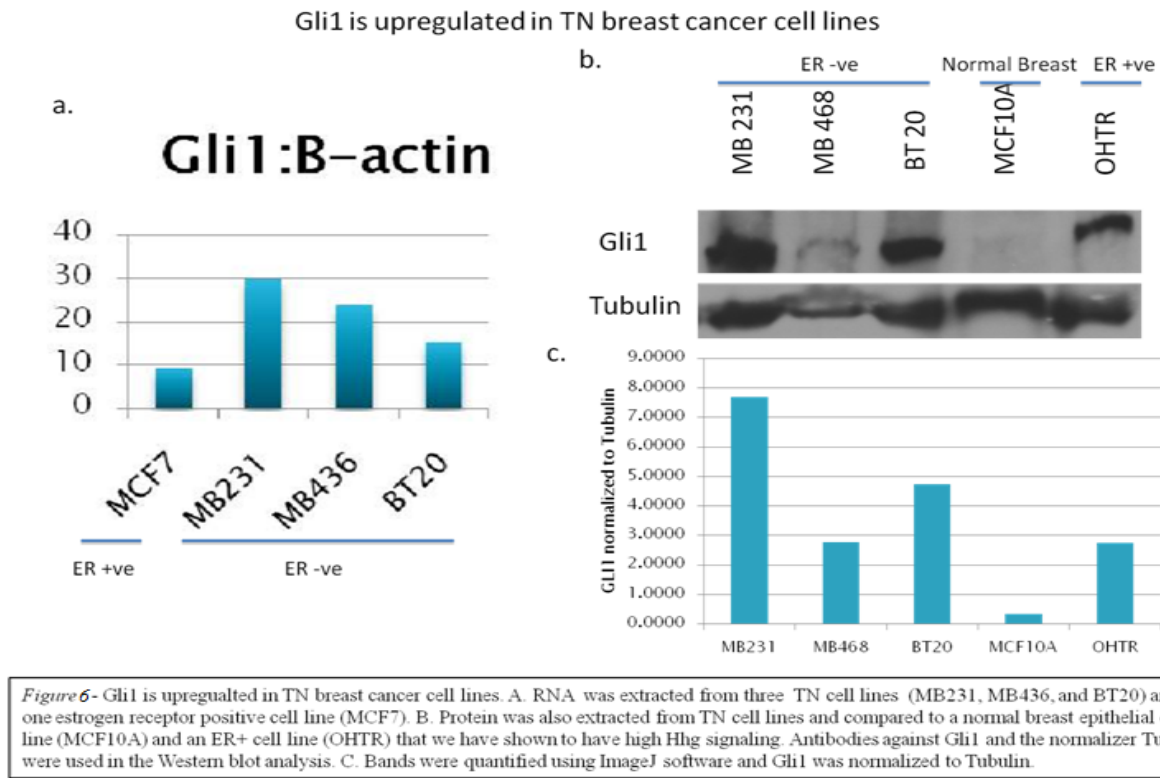


Figure 7

Effect of Gli1 expression on Disease Free Survival in ER α Negative Patients

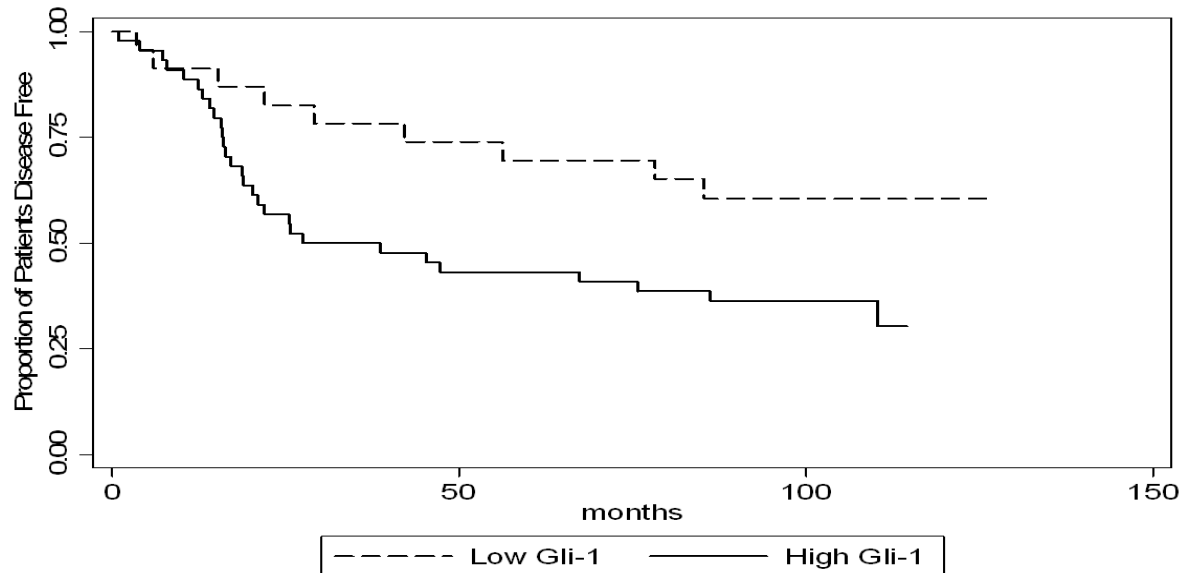


Figure 7- Kaplan-Meier disease free survival curve comparing low and high Gli1 expression status in ER α negative patient tumors. Patients with high Gli1 expression had much lower disease free survival than those with lower Gli1 expression [n=230].

Results

The PI3k/AKT pathways play important roles in Hedgehog signaling in TN Cell lines

The PI3k/AKT and MEK1/2 pathways are both known to play important roles in the activation of Hedgehog signaling in NIH3T3 cells. We assessed the effect of these pathways on Gli1 transcriptional activation by using an 8x Gli-luciferase plasmid assay. The plasmid constructs are shown in Figure 4 and described in the materials and method section. Cells were transfected with either the wild type or the mutant 8x Gli construct, media was changed 12 hours after transfection and then cells were treated with their respective drug. To assess individual

pathways effect, we used small molecule inhibitors specific for the respective pathways. LY2940002 is an established PI3K/AKT pathway inhibitor; U0126 is an inhibitor of the MEK1/2 pathways, while GDC-0449 is a drug in clinical trials for inhibiting the Hedgehog pathway by blocking SMO. After 36 hours of drug treatment the cells were harvested and luciferase expression analyzed. LY294002 inhibited Gli1 activation by ~ 50% while U0126 and GDC-0449 inhibited it by 30% (Figure 3). Luciferase expression from the mutant plasmid was not affected significantly, indicating the specificity of the assay and the drugs (data not shown).

To further characterize PI3k's affect on the Hhg pathway we transfected MB-231 cells with a series of PI3k and AKT expression vectors. We used a PI3k control vector (PBJ1), PI3k overexpression vector (P110* O/E), PI3k dominant negative vector (P110* K/R), AKT control vector (AKT-Con), AKT overexpression (AKT-myr O/E), and AKT dominant negative vector (AKT-K/R)³². To first test the extent of PI3k and AKT overexpression we isolated RNA after transfecting MB-231 cells and PCR amplified PI3k, AKT, and β -actin (loading control) transcripts. The PCR products were then separated on an agarose gel and quantified using ImageJ software (Figure 8a and 8b). The transfection efficiency of vectors expressing constitutively active and dominant negative AKT were very high but the PI3k was overexpressed at a moderate level. When cotransfected with the 8x Gli luciferase reporter plasmid in MB231 cells, the PI3k overexpression vector again showed moderate increase in Gli transcriptional activity. We are currently assaying the effects of AKT vectors on Gli transcriptional activity. This data suggests importance of PI3K/AKT pathway in regulating Hhg signaling in TN breast cancer and will continue to be investigated further.

Figure 8

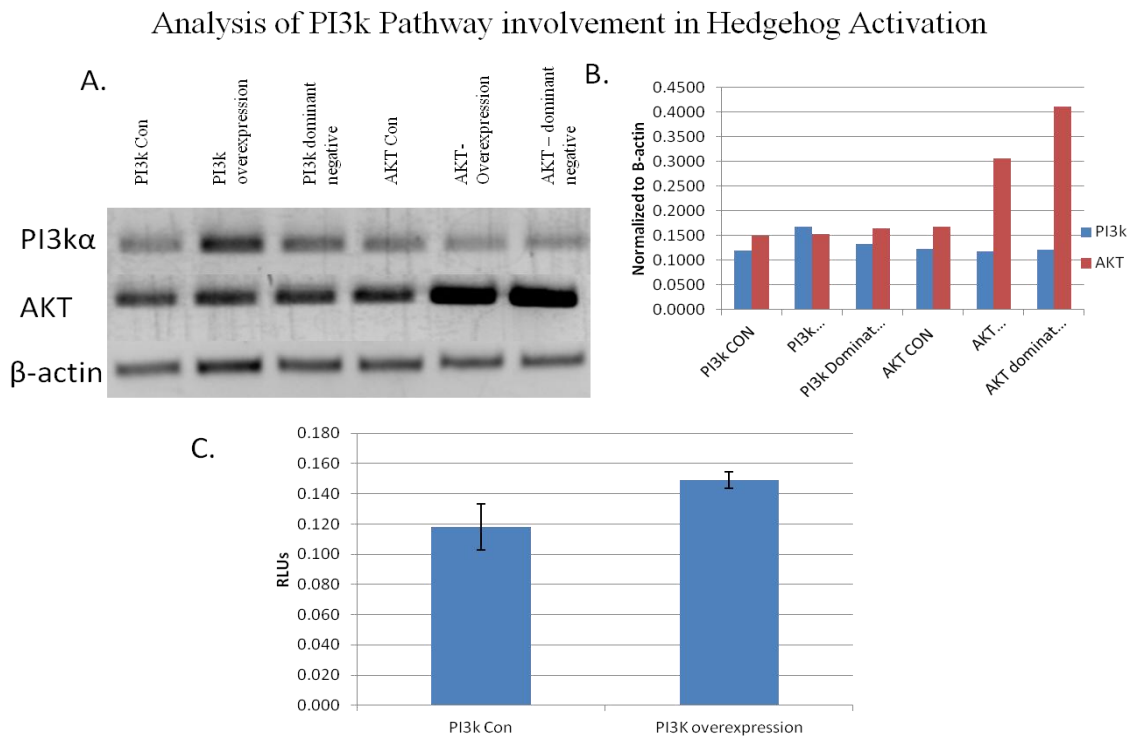


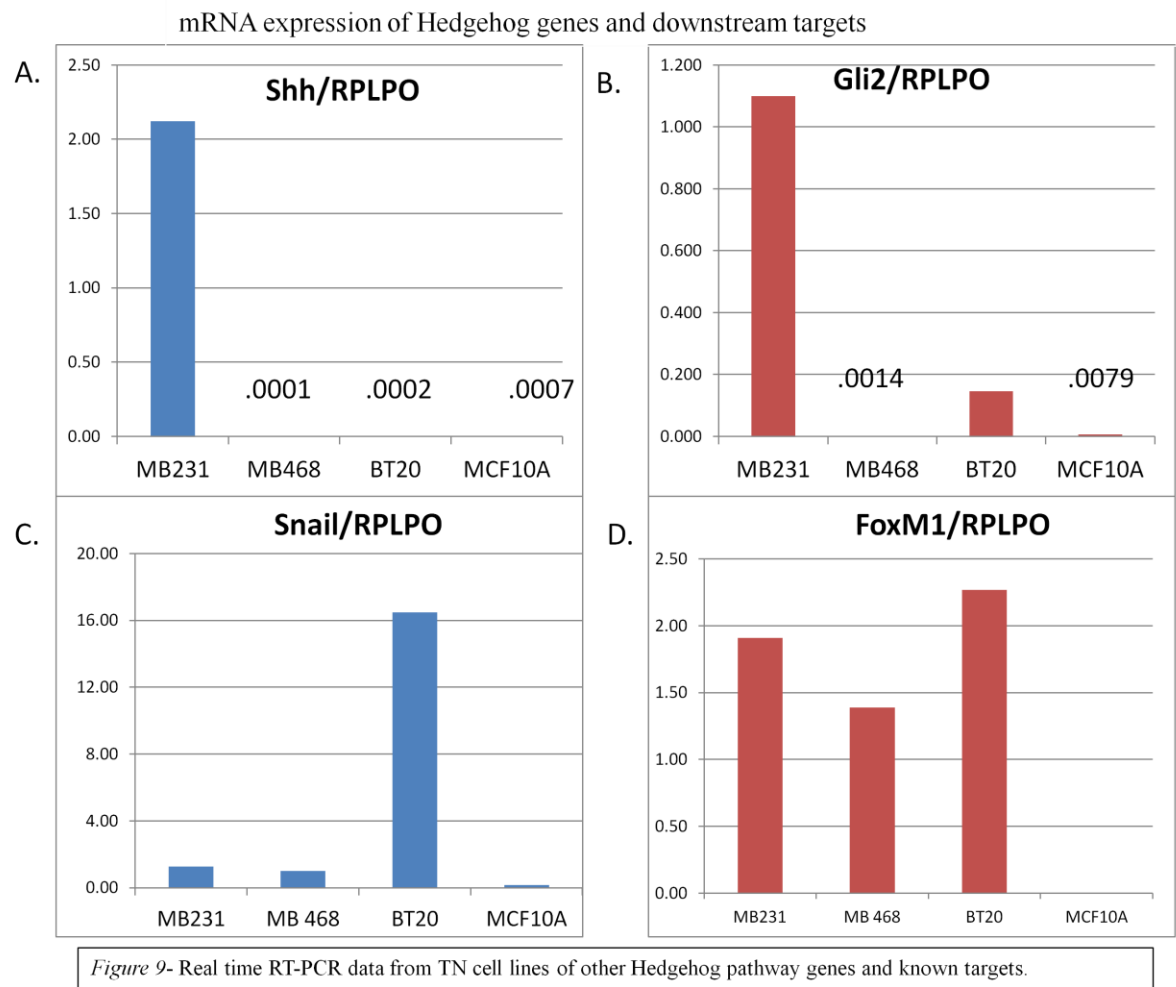
Figure 8- The PI3k pathway can activate Gli transcriptional activity. A. MB231 cells were transfected with PI3k Control (PBJ1), PI3k overexpression (P110 O/E), PI3k dominant negative (P110 K/R), AKT Control, AKT overexpression (AKT-myr), and AKT dominant negative (AKT K/R) plasmids. RNA was isolated from the cells and subjected to PCR amplification by PI3kα, AKT, and β-actin primers (available upon request). Products were run on an agarose gel. B. Gel products were quantified using ImageJ software. C. MB231 cells were cotransfected with the 8x-Gli luciferase plasmid, pRL-TK control plasmid, and either the PI3k control or overexpressing plasmid. Cells were harvested 36 hours after transfection and luciferase and renilla expression was quantified (Promega).*

Other Hedgehog Molecules and Gli1 Target Genes are upregulated in TN Cell lines

Total RNA from TN cell lines and MCF10A cells was isolated, subjected to Real-Time RT-PCR, and normalized to RPLPO. Sonic Hedgehog ligand (Shh) was seen to be high only in one TN cell lines, MB-231 (Figure 9a). This indicates the possibility that the Hhg pathway activation in some TN cells may not be occurring via canonical pathway activation. GLI2, which can function both as a transcriptional repressor and an activator, was upregulated in MB 231 and BT-20 TN cells but not MB-468 cells. Further work needs to be done to determine the role of GLI2 in these cells. Activation of other Hedgehog components except SMO and GLI1 seem to

be cell type specific. GLI1 transcriptional targets however were upregulated in TN cell lines when assayed by Real-time PCR. SNAIL1 and FOXM1 are both known targets³³ of Hhg pathway and were highly expressed in TN breast cancer cell lines when compared to normal breast epithelial MCF10A cells.

Figure 9



Effect of Anti-Hedgehog compounds alone and in combination with other targeted therapies on TN breast cancer cell lines:

With recent advances in the development of anti-Hhg compounds we wanted to test the therapeutic potential of targeting the Hhg pathway in TN cell lines. We tested two anti-Hhg compounds, Genentech's GDC-0449, a small molecule inhibitor of Smo currently in Phase III clinical trials for basal cell carcinoma, and the small molecule inhibitor GANT61 which inhibits the GLI transcription factors. We used MB 231 cells initially to test the efficacy of GDC-0449 alone in inhibiting cell proliferation rates but found the cells to be highly resistant to the drug [data not shown].

Based on our previous data we hypothesized the involvement of the PI3k pathway in activating Hhg signaling in TN breast cancer. We therefore tested the effect of combining PI3k and Hhg targeted therapies on these cells. LY294002 is a well established PI3k inhibitor commonly used *in vitro* and *in vivo* studies. We tested the effects of GDC0449 alone and in combination with LY294002 on MB231 cells in a MTT assay and using the 8x Gli luciferase reporter system. The MTT time course experiment showed relatively little growth inhibitory effects by GDC0449 (25uM) alone, but the LY294002 (20uM) alone showed significant growth inhibition (Figure 10a). When combined the two treatments resulting in further growth inhibition but not substantially different from the LY294002 alone treatment. The luciferase assay showed a similar pattern as inhibition of the GLI transcriptional activity was largely due to LY294002 and GDC0449 had a small contribution to inhibiting transcription (Figure 10b). Next we determined the effect of this combination therapy on MB-468 and MB-436 cell lines *in vitro*. We had seen in previous experiments that MB-468 cells were more sensitive to GDC0449 alone and decided to test two concentrations of the drug alone and in combination with LY294002 (Figure 10b). Again we saw significant inhibition of cell growth by LY294002 and a relatively minor effect of GDC-0449. This overall resistance to the anti-Smo compound, GDC0449, led us to

investigate if we could inhibit cell growth by targeting the Hh pathway further downstream of Smo.

For this purpose we tested the effect of GANT61, a potent GLI targeting small molecule, on the TN cell lines. We first treated MB-231 and MB-468 cells with varying concentrations of GANT61 (10 uM, 25 uM, and 50 uM) and found major inhibition of cell proliferation over a 72 hour period [Figure 11a and 11b]. Next we compared the effect of GANT61 alone and in combination with LY294002 to test the therapeutic potential of combined targeting of GLI and PI3K pathway (Figure 11c). GANT61 treatment alone resulted in ~40% inhibition of MB231 cell proliferation while there was ~25% inhibition in presence of LY294002. When combined together, GANT61 and LY294002 had an additive effect on cell proliferation resulting in ~60% inhibition. This data suggests that GANT61 could be a more potent inhibitor of the TN breast cancer cells compared to GDC0449.

Figure 10

TN cell lines treated with anti-Smo compound alone or in combination with a PI3k inhibitor

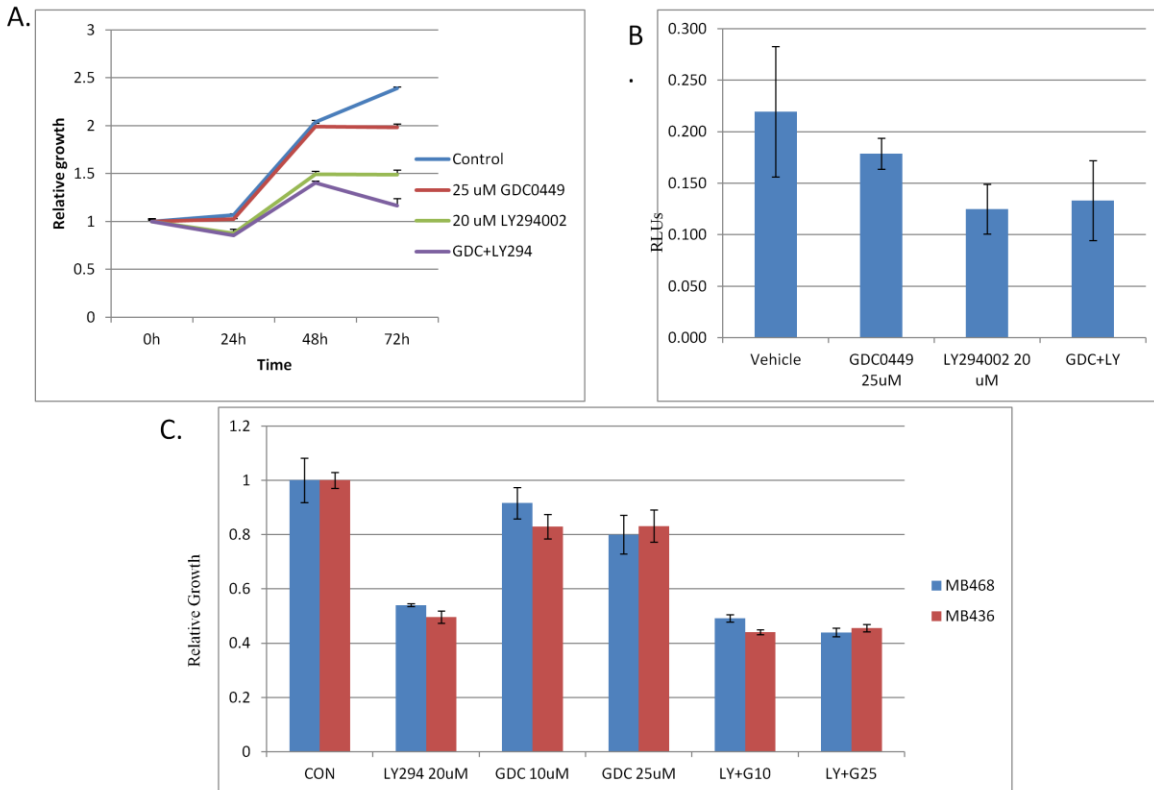
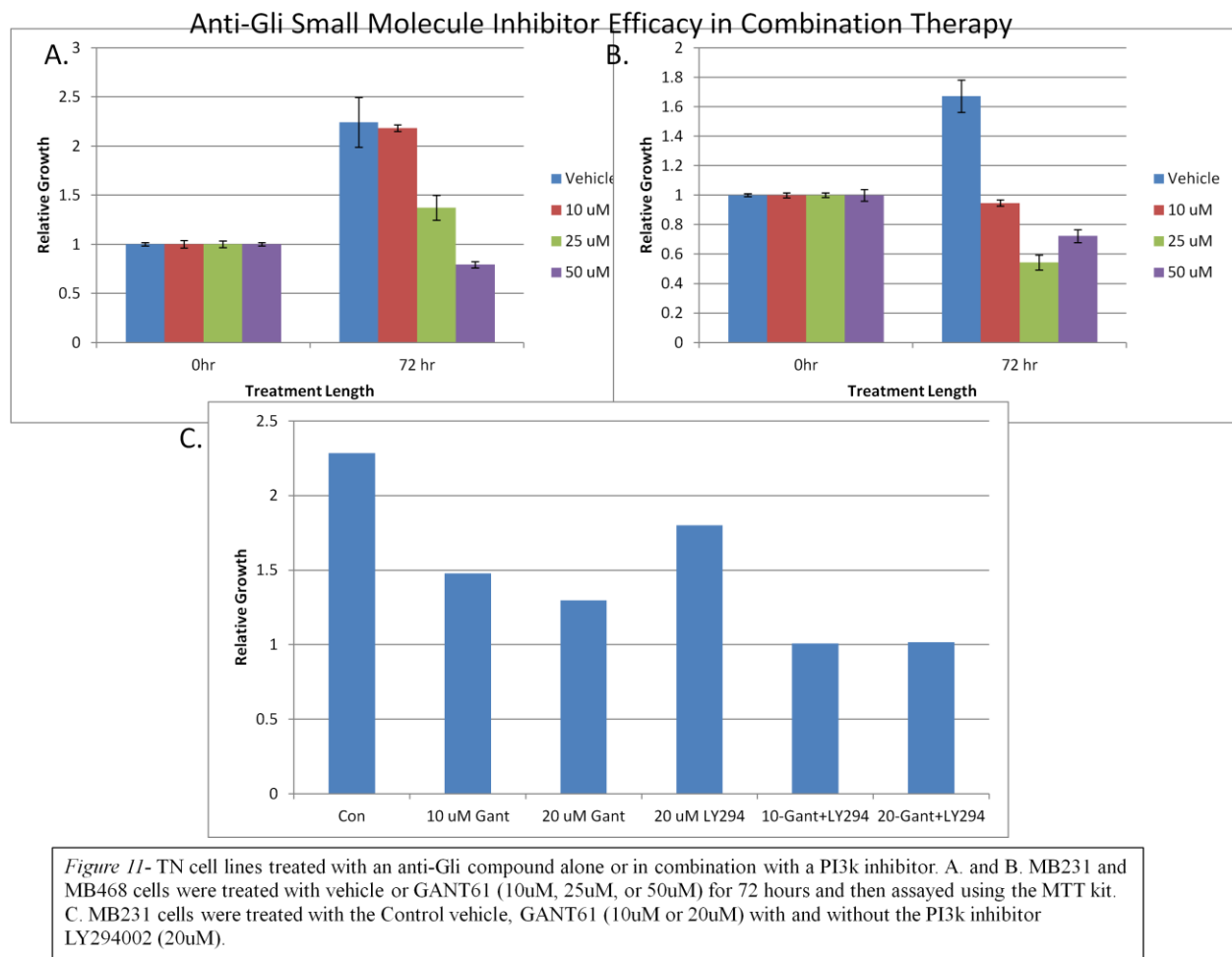


Figure 10- Anti-Smo and anti-PI3k combination therapy in TN cell lines. A. MB 231 MTT time course experiment. MB231 cells were treated with 20uM GDC0449, 20uM ly294002 or combined for 24, 48, and 72 hours. B. MB231 cells were transfected with 8xGli luciferase plasmid and then treated for 36 hours with GDC0449 (25uM), LY294002 (20uM), or both. B. MB 468 and MB436 cells were treated for 72 hours with either LY294002 (20uM), GDC0449 (10uM or 25uM), or both. Following completion of the 72 hours, cell viability was assayed using the MTT kit from Roche and absorbance measured.

Figure 11



Discussion

Our data indicates that the Hedgehog pathway is activated in triple negative breast cancer cells and that this activation results from cross-talk with other signaling pathways activated during carcinogenesis. The key signaling molecules in Hedgehog pathway, SMO and GLI1, are upregulated in triple negative cell lines. Other groups have shown the importance of the pathway by knocking down Gli1 in MB231 cells and seeing a significant growth inhibition compared to the control cells¹³.

We investigated the mechanism behind upregulation of the Hhg pathway and our data suggests the importance of other cell signaling pathways in regulating the Hedgehog pathway. Using an 8x Gli-Luciferase reporter assay we have showed the importance of the MEK1/2 pathways but also the PI3K/AKT pathways, suggesting the involvement of other signaling pathways in Hedgehog activation. We also used PI3k overexpression vector in conjunction with the luciferase reporter system to show that constitutively active PI3k activates GLI transcriptional activity. Further investigation of the mechanism involved is currently underway.

Lastly, we tested the possible therapeutic implications of targeting the Hhg pathway in TN breast cancer. We used two anti-Hhg compounds that target different proteins in the Hhg pathway, which yielded very different results. Most TN cells, especially MB231, were markedly resistant to GDC-0449, the Smo inhibitor that is being tested in Phase III clinical trials in basal cell carcinoma. We next looked at a combination therapy based approach utilizing our observation that the PI3k pathway is important for GLI activation. We used the common PI3k inhibitor LY294002 alone and in combination with GDC0449 and found most of the inhibitory effects on GLI activation and cell proliferation were due predominantly to LY294002. The mechanism contributing the resistance to GDC0449 remains unclear and is an area of future investigation for us.

Noting the resistance to the upstream protein Smo, we decided to test an inhibitor of Hhg that targets the pathway further downstream. GANT61 specifically targets the Gli1 transcription factors, blocking major transcriptional activity of the Hhg pathway. We tested this compound alone and in combination with LY294002 on cell growth. GANT61 showed a much higher potency against MB-231 and MB-468 cells compared to GDC-0449. When compared to the PI3k inhibitor, LY294002, we found the GANT61 to have higher efficacy in inhibiting MB-231 cells

compared to LY294002, and the combination had an additive effect. Although GANT61 is not in clinical trials, a strategy to inhibit GLI1 could be a potential option to inhibit Hh pathway. Our studies clearly shows that therapeutic targeting of the Hh pathway, especially in combination with PI3k pathway could be a rationale approach in treating patients with TN breast cancer. With GDC-0449 already approved for clinical use, it is important for us to find the mechanism underlying reduced response of the TN cells to SMO inhibitors.

There is a critical need to both understand and to develop new therapies for triple negative breast cancer. Our studies here show that the Hedgehog pathway is activated in triple negative breast cancer and that this activation may be in part due to other signaling pathways (summarized with inhibitors in figure 12). We show here the therapeutic potential *in vitro*, as well as some of the difficulty in targeting the pathway. Future studies will determine the importance of the downstream target genes of the active GLI transcription factors, validate the mechanism behind its upregulation, and test the efficacy of anti-Hedgehog therapeutics *in vivo*.

Figure 12

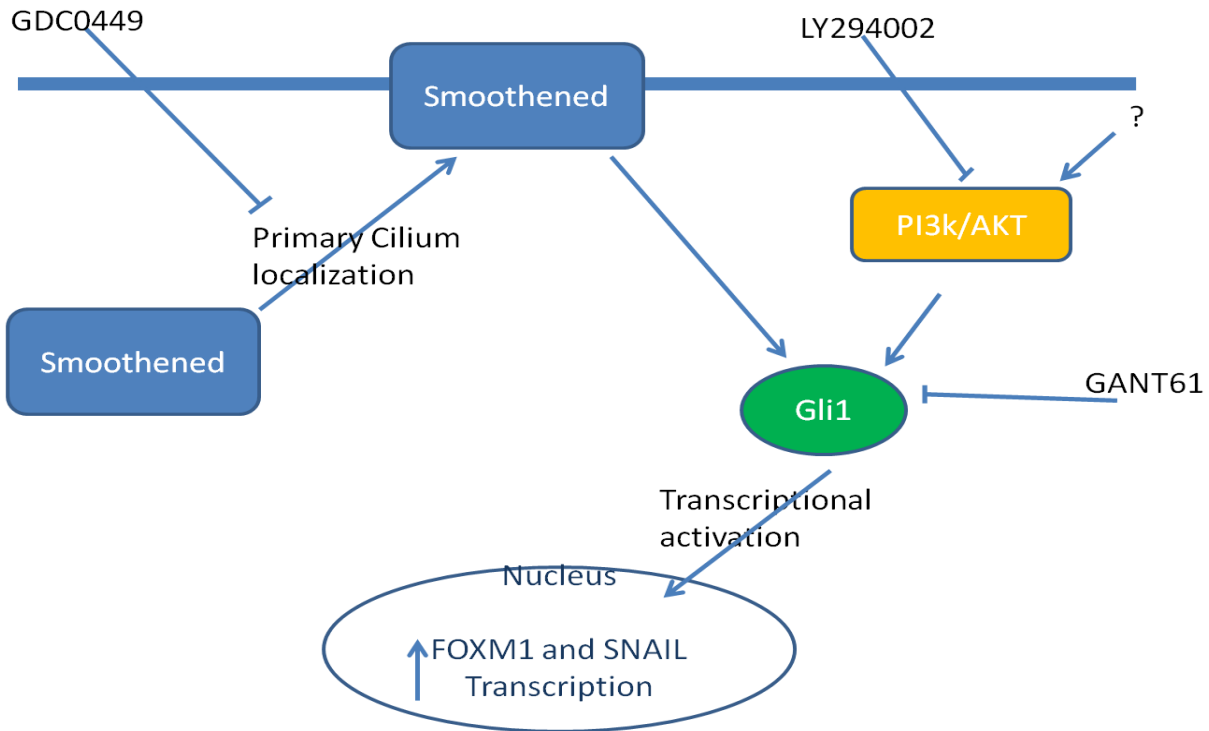


Figure 12- Schematic of HhG activation and therapeutic targeting in triple negative cell lines.

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References

1. Campbell JB: Breast cancer-race, ethnicity, and survival: a literature review. *Breast Cancer Research & Treatment* 74:187-92, 2002
2. American Cancer Society. Surveillance Research 2011.
<<http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-030975.pdf> >
3. Rakha EA, Reis-Filho JS and Ellis IO. Basal-like breast cancer: a critical review. *J. Clin. Oncol.* 2008;26:2568–81.
4. Khalifeh IM, Albarracin C, Diaz LK, et al. Clinical, histopathologic, and immunohistochemical features of microglandular adenosis and transition into in situ and invasive carcinoma, *Am. J. Surg. Pathol.* 2008;32:544–52.
5. Varjosalo M and Taipale J. Hedgehog: functions and mechanisms. *Genes Dev.*, 22 (2008), pp. 2454-247.
6. Sasaki H, Nishizaki Y, Hui C, et al. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* 1999; 126: 3915-24.
7. Teglund S, Toftgard R. Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim Biophys Acta* 2010;1805:181–208.

8. Chen JQ and Russo J. ER α -negative and triple negative breast cancer: Molecular features and potential therapeutic approaches. *Biochimica et Biophysica Acta- Reviews on Cancer*. 2009;2:162-75.
9. Medscape Education Oncology. "Hedgehog Pathway Inhibitors: Emerging targets for cancer therapy." Medscape. 2011. Apr 1, 2012. <<http://www.medscape.org/viewarticle/750440>>
10. Kasper M, Jaks V, Fiaschi M, et al. Hedgehog signaling in breast cancer. *Carcinogenesis*. 2009 Jun;30(6):903-11.
11. Xu L, Kwon YJ, Frolova N, et al. Gli1 promotes cell survival and is predictive of a poor outcome in ER α -negative breast cancer. *Breast Cancer Res Treat*. 2010 Aug;123(1):59-71.
12. Moran CM, Myers CT, Lewis CM, Krieg PA. Hedgehog regulates angiogenesis of intersegmental vessels through the VEGF signaling pathway. *Dev Dyn*. 2012 Apr 18.
13. Kwon YJ, Hurst DR, Steg AD, Yuan K, Vaidya KS, Welch DR, Frost AR. Gli1 enhances migration and invasion via up-regulation of MMP-11 and promotes metastasis in ER α negative breast cancer cell lines. *Clin Exp Metastasis*. 2011. Jun;28(5):437-49.
14. Rubin LL, de Sauvage FJ. Targeting the Hedgehog pathway in cancer. *Nat Rev Drug Discov* 2006; 5: 1026-33.
15. Hahn H, Wicking C, Zaphiropoulos PG, et al. Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. *Cell* 1996; 85: 841-51.
16. Lewis MT, Ross S, Strickland PA, et al. Defects in mouse mammary gland development caused by conditional haploinsufficiency of Patched-1. *Development* 1999; 126: 5181-93.

17. Sims-Mourtada J, Izzo JG, Ajani J, et al. Sonic Hedgehog promotes multiple drug resistance by regulation of drug transport. *Oncogene* 2007; 26: 5674-9.
18. Hassounah NB, Bunch TA, McDermott KM. Molecular Pathways: The Role of Primary Cilia in Cancer Progression and Therapeutics with a Focus on Hedgehog Signaling. *Clin Cancer Res.* 2012 Apr 18.
19. Huangfu D, Liu A, Rakeman AS, et al. Hedgehog signaling in the mouse requires intraflagellar transport proteins. *Nature* 2003; **426**:83–7.
20. Huangfu D, Anderson KV. Cilia and Hedgehog responsiveness in the mouse. *Proc Natl Acad Sci U S A* 2005; **102**:11325–30.
21. Schraml P, Frew IJ, Thoma CR, Boysen G, Struckmann K, Krek W, et al. Sporadic clear cell renal cell carcinoma but not the papillary type is characterized by severely reduced frequency of primary cilia. *Mod Pathol* 2009;22:31–6.
22. Tukachinsky H, Lopez LV, Salic A. A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. *J Cell Biol* 2010;191:415–28.
23. Nachury MV, Seeley ES, Jin H. Trafficking to the ciliary membrane: how to get across the periciliary diffusion barrier? *Annu Rev Cell Dev Biol* 2010;26:59–87
24. Rohatgi R, Milenkovic L, Scott MP. Patched1 regulates hedgehog signaling at the primary cilium. *Science* 2007;317:372–6.

25. Seeley ES, Carriere C, Goetze T, Longnecker DS, Korc M. Pancreatic cancer and precursor pancreatic intraepithelial neoplasia lesions are devoid of primary cilia. *Cancer Res* 2009;69:422–30.
26. Yuan K, Frolova N, Xie Y, Wang D, Cook L, Kwon YJ, et al. Primary cilia are decreased in breast cancer: analysis of a collection of human breast cancer cell lines and tissues. *J Histochem Cytochem* 2010; 58:857–70.
27. Riobó NA, Lu K, Ai X, Haines GM, Emerson CP Jr. Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. *Proc Natl Acad Sci U S A*. 2006 Mar 21;103(12):4505-10.
28. Riobo NA, Haines GM, Emerson CP Jr. Protein kinase C-delta and mitogen-activated protein/extracellular signal-regulated kinase-1 control GLI activation in hedgehog signaling. *Cancer Research* 2006; 66(2):839-45.
29. Von Hoff DD, LoRusso PM, Rudin CM, et al. Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. *N Engl J Med*. 2009 Sep 17;361(12):1164-72.
30. Mazumdar T, Devecchio J, Agyeman A, Shi T, Houghton JA. Blocking Hedgehog survival signaling at the level of the GLI genes induces DNA damage and extensive cell death in human colon carcinoma cells. *Cancer Res*. 2011 Sep 1;71(17):5904-14.
31. Sasaki H, Hui C, Nakafuku M, Kondoh H. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development*. 1997 Apr;124(7):1313-22.
32. Datta J, Majumder S, Kutay et al. Metallothionein expression is suppressed in primary human hepatocellular carcinomas and is mediated through inactivation of CCAAT/enhancer

binding protein alpha by phosphatidylinositol 3-kinase signaling cascade. *Cancer Res.* 2007 Mar 15;67(6):2736-46.

33. Sirohi B, Arnedos M, Popat S, et al. Platinum-based chemotherapy in triple-negative breast cancer. *Ann Oncol* 2008;19:1847–52.